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Original Article

Upregulation of miR-202-5p promotes cell apoptosis and suppresses cell viability of hypoxia-induced myocardial H9c2 cells by targeting SOX6 to inhibit the activation of the PI3K/AKT/FOXO3a pathway

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Abstract: This study aimed to investigate the potential role of microRNA-202-5p (miR-202-5p) in regulating myocardial ischemia-caused injury and to explore the underlying mechanisms. Rat embryonic ventricular cardiomyocyte-derived H9c2 cells were treated with hypoxia to generate an *in vitro* myocardial ischemia model, followed by the transfection with a miR-202-5p mimic and inhibitor. Subsequently, the effects of miR-202-5p on cell viability, apoptosis, migration, and invasion were analyzed. A luciferase reporter assay was used to identify the target gene of miR-202-5p. Besides, the regulatory relationship between miR-202-5p and the PI3K/AKT/FOXO3a pathway was investigated in hypoxia-induced H9c2 cells. Compared to normal H9c2 cells, the hypoxia treatment resulted in a significant damage to H9c2 cells, thereby decreasing the cell viability, migration, and invasion ability and inducing the cell apoptosis. miR-202-5p was significantly upregulated in hypoxia-induced H9c2 cells. After cell transfection, the suppression of miR-202-5p significantly alleviated the hypoxia-induced damage in H9c2 cells through the suppression of cell apoptosis and the promotion of cell viability, migration, and invasion ability. SRY-box 6 (SOX6) was found to be a direct target of miR-202-5p. The knockdown of SOX6 significantly aggravated the hypoxia-induced myocardial damage to H9c2 cells, which was alleviated after the inhibition of miR-202-5p expression. Besides, miR-202-5p suppression resulted in the activation of the PI3K/AKT/FOXO3a pathway in H9c2 cells. The data presented in this study revealed that miR-202-5p was upregulated in H9c2 cells during myocardial ischemia. The overexpressed miR-202-5p may aggravate the myocardial ischemia-caused injury by downregulating SOX6 to suppress the activation of the PI3K/AKT/FOXO3a pathway. Thus, miR-202-5p may serve as a potential target for the clinical treatment of myocardial ischemia.

Keywords: MicroRNA-202-5p, myocardial ischemia, SOX6, PI3K/AKT/FOXO3a pathway

Introduction

Myocardial infarction represents the death of cardiomyocytes, caused by extended ischemia [1]. Acute myocardial infarction is a common heart disease with a high mortality worldwide [2, 3]. Ischemia and reperfusion (I/R) injury is a common result of acute myocardial infarction, and preventing myocardial I/R injury is considered a promising therapeutic strategy for the treatment of acute myocardial infarction [4, 5]. Therefore, a better understanding of the key mechanism involved in myocardial ischemia will facilitate the development of therapies for myocardial infarction.

MicroRNAs (miRNAs), small non-coding RNA molecules, have emerged as key players in a wide range of biological functions and cellular processes, owing to their ability to negatively regulate gene expression [6, 7]. Several miRNAs, such as miR-1, miR-21, miR-29, miR-92a, miR-133, miR-199a, and miR-320, have been found to modulate I/R injury and/or remodeling

after myocardial infarction [8]. miRNAs are also regarded as promising biomarkers and therapeutic targets for acute myocardial ischemia [9]. Recently, the biological function and pathological roles of miR-202-5p in diseases have attracted much attention. For instance, miR-202 can inhibit hepatocellular carcinoma cell proliferation through targeting low-density lipoprotein receptor-related protein 6 (LRP6) [10]. miR-202 also suppresses the osteosarcoma cell proliferation and induces the apoptosis via targeting transcription factor GLI family zinc finger 2 (GLI2) [11]. miR-202 can induce the cell apoptosis in esophageal squamous cell carcinoma by regulating HSF2 [12]. Notably, a recent study has revealed that miR-202-5p is upregulated in a rat model of post-infarction heart failure [13]. Nevertheless, it is largely unknown whether miR-202-5p is involved in myocardial ischemia-caused damage.

In this study, rat embryonic ventricular cardiomyocyte-derived H9c2 cells were treated with hypoxia to generate an *in vitro* myocardial ischemia model. The expression and potential roles of miR-202-5p were then investigated in hypoxia-induced H9c2 cells. We aimed to explore the role and potential regulatory mechanism of aberrant expression of miR-202-5p in myocardial ischemia-caused damage to better understand the molecular mechanism of myocardial injury.

Materials and methods

Cell culture, treatment, and transfection

Rat embryonic ventricular cardiomyocyte-derived H9c2 cells (ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin in an incubator with 95% air and 5% CO_2 at 37°C. To simulate the hypoxia, H9c2 cells were cultured under hypoxic conditions in an atmosphere containing 94% N_2, 5% CO_2, and 1% O_2.

In cell transfection experiments, H9c2 cells were transfected with a miR-202-5p mimic, miR-202-5p inhibitor, their respective negative controls (NCs) (Life Technologies, Carlsbad, CA, USA), small interfering RNA (siRNA) against SRY-box 6 (SOX6), and control siRNA (Life Technologies) for 72 h using the Lipofectamine® RNAiMAX transfection reagent (Life Technologies) following the protocol recommended by the manufacturer.

Cell viability assay

Cell viability was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2 5-diphenyl-2*H*-tetrazolium bromide (MTT) colorimetric assay. In brief, cells from different treatment groups were seeded into 96-well plates and incubated for 24 h. Then, 20 μL of MTT was added into each well, and incubation continued for 4 h. Afterward, 150 μL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals for 10 min. The absorbance of each well was determined at 570 nm using an absorption spectrophotometer (Olympus, Japan). Each determination was performed three times.

Migration and invasion assays

To determine the cell migration ability, a modified two-chamber migration assay was performed. Briefly, cells were suspended in 200 μL of serum-free medium and plated into the upper compartment of a 24-well Transwell culture chamber with a pore size of 8 μm. Complete medium (600 μL) was added to the lower compartment of the chamber. After incubation for 48 h at 37°C, the cells were fixed with methanol. After removing non-traversed cells present on the upper surface with a cotton swab, traversed cells were stained on the lower surface with crystal violet and counted under a light microscope (Axio Observer 3m, Olympus, Japan).

For cell invasion, BD BioCoat™ Matrigel™ invasion chambers with an 8-μm pore size (BD Biosciences) were used. Briefly, the cells suspended in 200 μL of serum-free DMEM were seeded in the upper compartment of invasion chambers, while complete medium containing 10% FBS was added to the lower compartment. After incubation for 48 h, non-invaded cells were carefully removed with a cotton swab, and invaded cells, attached to the bottom surface, were fixed with 100% methanol, stained with a crystal violet solution, and counted under a light microscope (Axio Observer 3m).

Apoptosis detection by flow cytometry

Cell apoptosis was detected by flow cytometry analysis after double staining with propidium

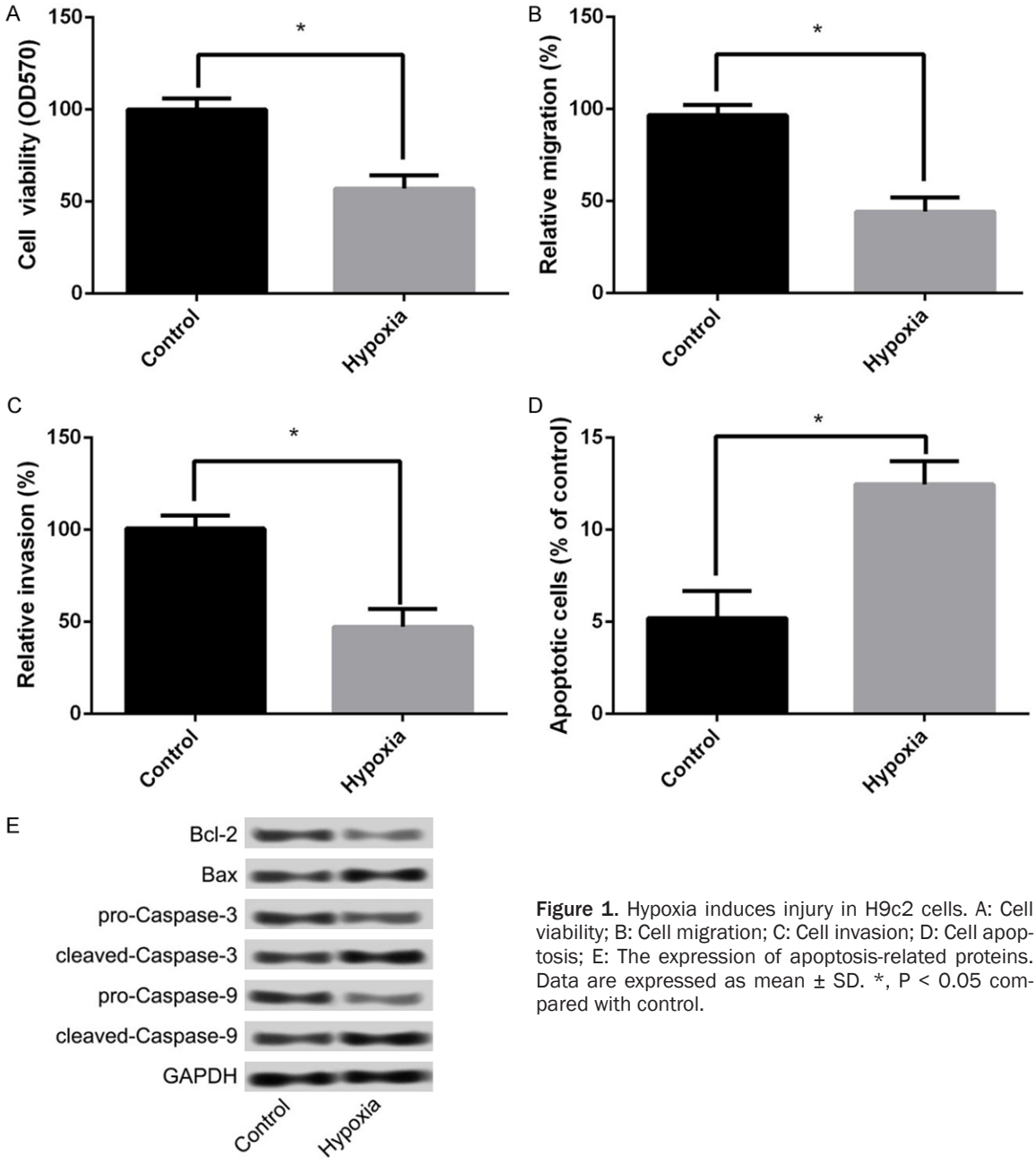


Figure 1. Hypoxia induces injury in H9c2 cells. A: Cell viability; B: Cell migration; C: Cell invasion; D: Cell apoptosis; E: The expression of apoptosis-related proteins. Data are expressed as mean ± SD. *, $P < 0.05$ compared with control.

iodide (PI) and fluorescein isothiocyanate (FITC)-conjugated annexin V. Briefly, the cells from different treatment groups were fixed in 70% ethanol, stained with PI/FITC-annexin V in the presence of 50 μg/mL RNase A (Sigma-Aldrich), and then incubated at room temperature for 1 h away from light. Flow cytometry analysis for detecting cell apoptosis was conducted using a FACScan (Beckman Coulter, Fullerton, CA, USA), and the percentage of apoptotic cells was analyzed using the FlowJo software (TreeStar, Ashland, OR, USA).

Luciferase reporter assay

To construct the reporter vector SOX6-wild-type (SOX6-WT), a fragment of the *SOX6* gene containing the predicted miR-202-5p binding site was amplified by PCR and then cloned into the pmirGlO dual-luciferase miRNA target expression vector (Promega, Madison, WI, USA). The sequence of the putative binding site of miR-202-5p in the *SOX6* gene was replaced to obtain the reporter vector SOX6-mutated-type (SOX6-MUT). Then, the reporter vectors (SOX6-

WT or SOX6-MUT) and the miR-202-5p mimic were co-transfected into HEK 293T cells, and their luciferase activities were determined using a dual-luciferase reporter assay system (Promega).

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cells using the TRIzol reagent (Life Technologies). After assessing the quality of total RNA using a SMA 400 UV-VIS spectrophotometer (Merinton, Shanghai, China), real-time PCR was performed using the One-Step SYBR® PrimeScript® PLUS RT-PCR kit (Takara Biotechnology, Dalian, China) to determine the relative expression levels of SOX6, PI3K, AKT, and FOXO3a. PCR with the TaqMan microRNA reverse transcription kit, TaqMan universal master mix II, TaqMan microRNA assay of miR-202-5p and U6 (Applied Biosystems, Foster City, CA, USA) was used to determine the relative expression levels of miR-202-5p. GAPDH and U6 expression levels were, respectively, used to normalize the expression levels of mRNAs or miRNAs. Fold changes were calculated according to cycle threshold (Ct) values using the $2^{-\Delta\Delta Ct}$ method. Each reaction was carried out in triplicate.

Western blot assay

Total proteins were extracted from cells using the RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) containing protease inhibitors (Roche, Guangzhou, China) and then quantified with a BCA™ protein assay kit (Pierce, Appleton, WI, USA). The western blot assay for detecting the expression of proteins was carried out with a Bio-Rad Bis-Tris gel system following the manufacturer's protocols. Separated proteins were transferred onto polyvinylidene difluoride membranes. Primary antibodies to BCL-2, BAX, pro-caspase-3, cleaved caspase-3, pro-caspase-9, cleaved caspase-9, p-PI3K, PI3K, p-AKT, AKT, FOXO3a (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and SOX6 (1:2,000, Abcam, USA) were prepared in 5% blocking buffer and then incubated with the membranes at 4°C overnight. The membranes were rinsed three times and then incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h. The membrane blots were rinsed, then covered with the Immobilon western chemiluminescent HRP substrate (Millipore, MA, USA), and the protein bands were visualized using a Bio-Rad ChemiDoc™ XRS system. The intensity of protein bands was quantified using the Image Lab™ software (Bio-Rad, Shanghai, China).

Statistical analysis

All experiments were repeated three times, and the data were expressed as the mean ± SD. Differences between groups were compared by one-way analysis of variance using the GraphPad 6.0 statistical software (GraphPad Software, San Diego, CA, USA). A value of $P < 0.05$ indicated a statistically significant difference.

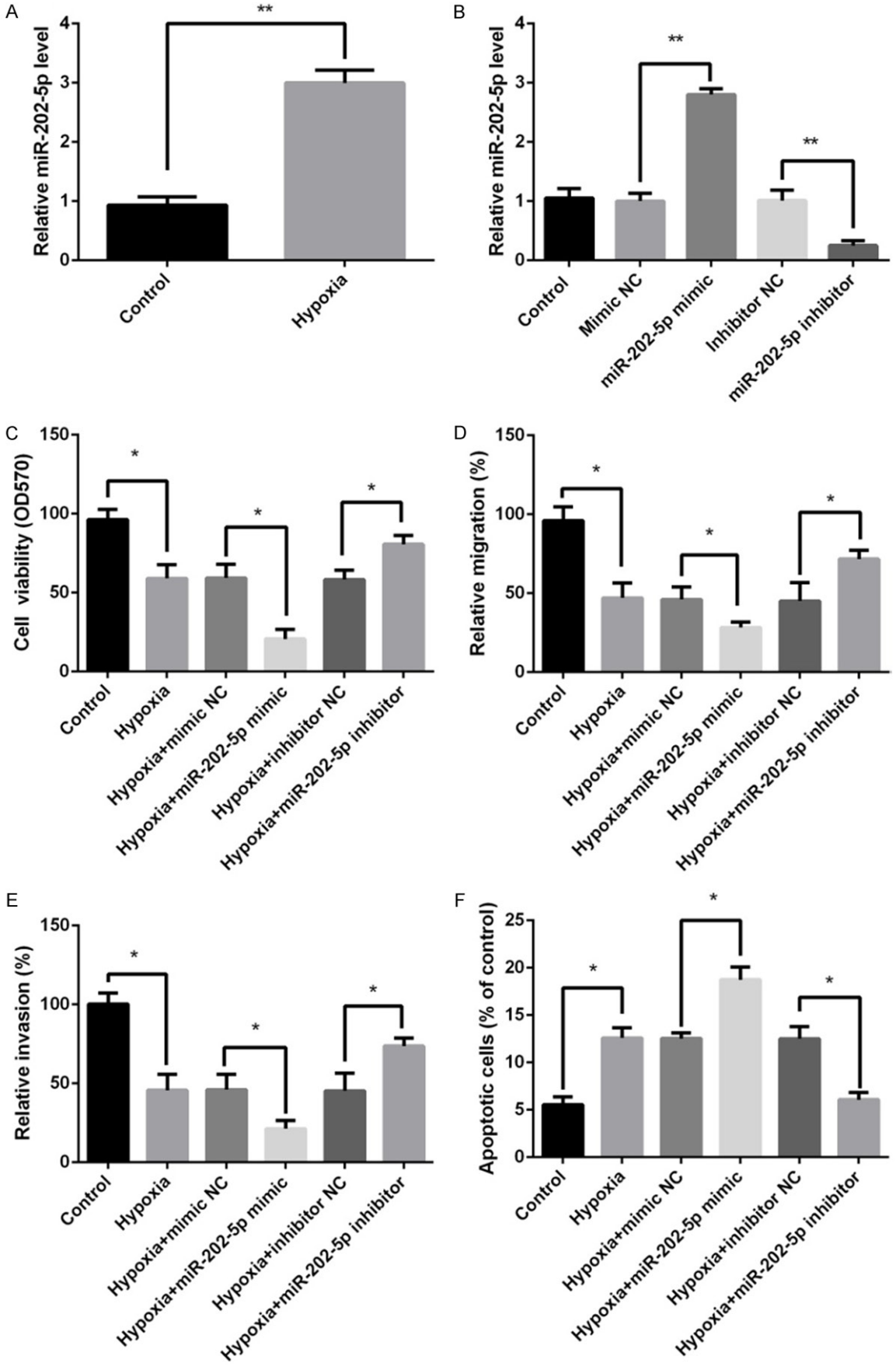
Results

Hypoxia-induced injury in H9c2 cells

We first constructed an in vitro myocardial ischemia model using H9c2 cells treated under hypoxia. Compared to those in the control cells, the cell viability ($P < 0.05$), migration ($P < 0.05$), and invasion ability ($P < 0.05$) of H9c2 cells were all significantly decreased by the hypoxia treatment (**Figure 1A-C**), while the cell apoptosis was markedly increased ($P < 0.05$; **Figure 1D**, **1E**). Compared with those in the control cells, cell apoptosis-related proteins such as BCL-2, pro-caspase-3, and pro-caspase-9 were significantly decreased, while the expression of BAX, active caspase-3, and active caspase-9 was markedly increased by the hypoxia treatment ($P < 0.05$; **Figure 1E**). These results indicated the damages to H9c2 cells caused by hypoxia treatment.

Suppression of miR-202-5p alleviated hypoxia-induced myocardial damage

After constructing the cell model of myocardial ischemia in vitro, we analyzed the expression of miR-202-5p. The results showed that miR-202-5p significantly increased in hypoxia-treated cells compared to its expression in control cells ($P < 0.01$; **Figure 2A**), implying a correlation between miR-202-5p abnormal expression and H9c2 cell damage. Furthermore, we transfected the vectors carrying the miR-202-5p mimic, inhibitor, and their corresponding NCs into hypoxia-induced H9c2 cells. The results showed that the expression of miR-202-5p was significantly increased by mimic transfection, while markedly decreased by inhibitor transfec-


A
Relative miR-202-5p level
**
Control
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B
Relative miR-202-5p level
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Control
Mimic NC
miR-202-5p mimic
Inhibitor NC
miR-202-5p inhibitor
C
Cell viability (OD570)
*
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Control
Hypoxia
Hypoxia+mimic NC
Hypoxia+miR-202-5p mimic
Hypoxia+inhibitor NC
Hypoxia+miR-202-5p inhibitor
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Control
Hypoxia
Hypoxia+mimic NC
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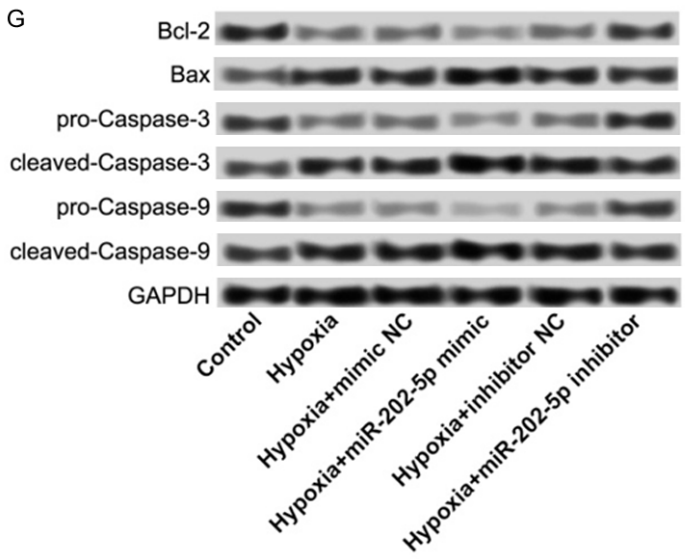

Figure 2. miR-202-5p was upregulated under hypoxic conditions and the suppression of miR-202-5p alleviated hypoxia-induced myocardial damage. A: The expression of miR-202-5p in control and hypoxia treated cells. B: The expression of miR-202-5p in different treatment groups; C: Cell viability; D: Cell migration; E: Cell invasion; F: Cell apoptosis; G: The expression of apoptosis-related proteins. Data are expressed as mean ± SD. *, P < 0.05, **, P < 0.01.

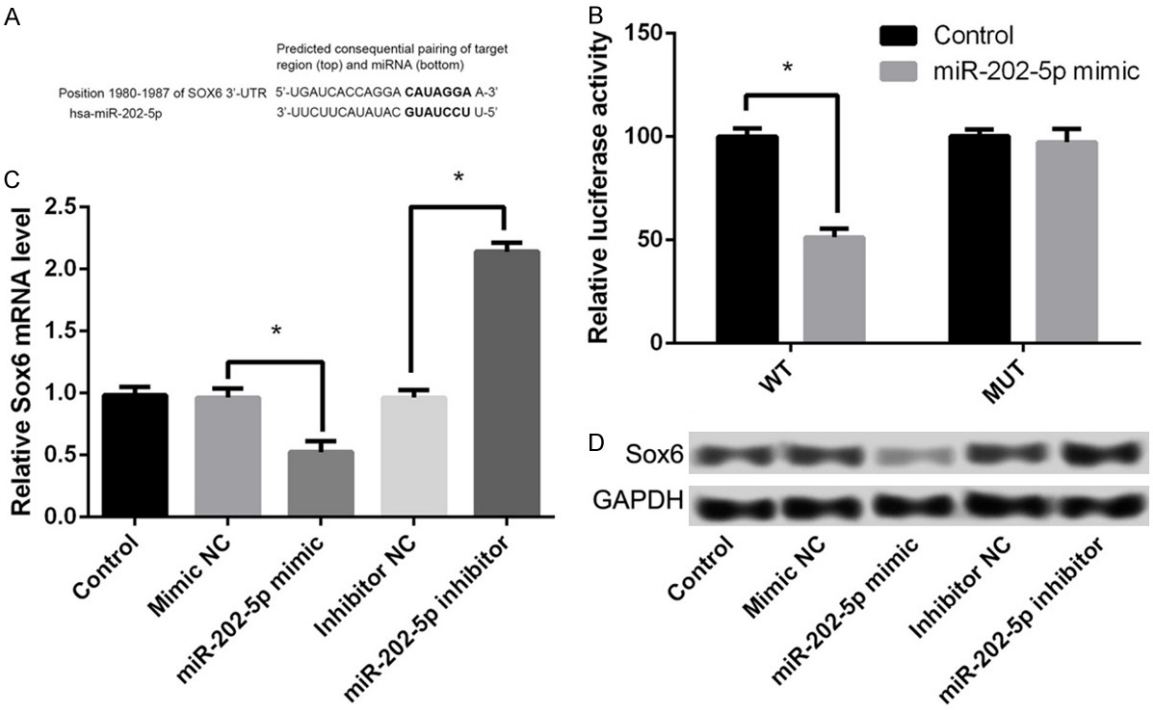

Figure 3. SOX6 was a target of miR-202-5p and its expression was negatively regulated by miR-202-5p. A: The predicted binding sequence of SOX6 and miR-202-5p. B: The luciferase report activity of SOX6-WT and SOX6-MUT. C and D: The mRNA and protein expression of SOX6 in different miRNAs transfection groups. Data are expressed as mean ± SD. *, P < 0.05, **, P < 0.01.

tion (P < 0.01; **Figure 2A**), suggesting that the transfection was successful. Moreover, the cell viability, migration, and invasion ability of hypoxia-induced H9c2 cells significantly increased after transfection with the miR-202-5p inhibitor (P < 0.05; **Figure 2C-E**). Additionally, the percentage of apoptotic cells and the levels of BAX/BCL-2, active caspase-3, and active caspase-9 were significantly decreased by miR-202-5p inhibitor transfection (P < 0.05; **Figure 2F**, **2G**). Opposite effects on the cell viability, migration, invasion, and apoptosis were observed after miR-202-5p mimic transfection (P < 0.05; **Figure 2C-G**). These results revealed that

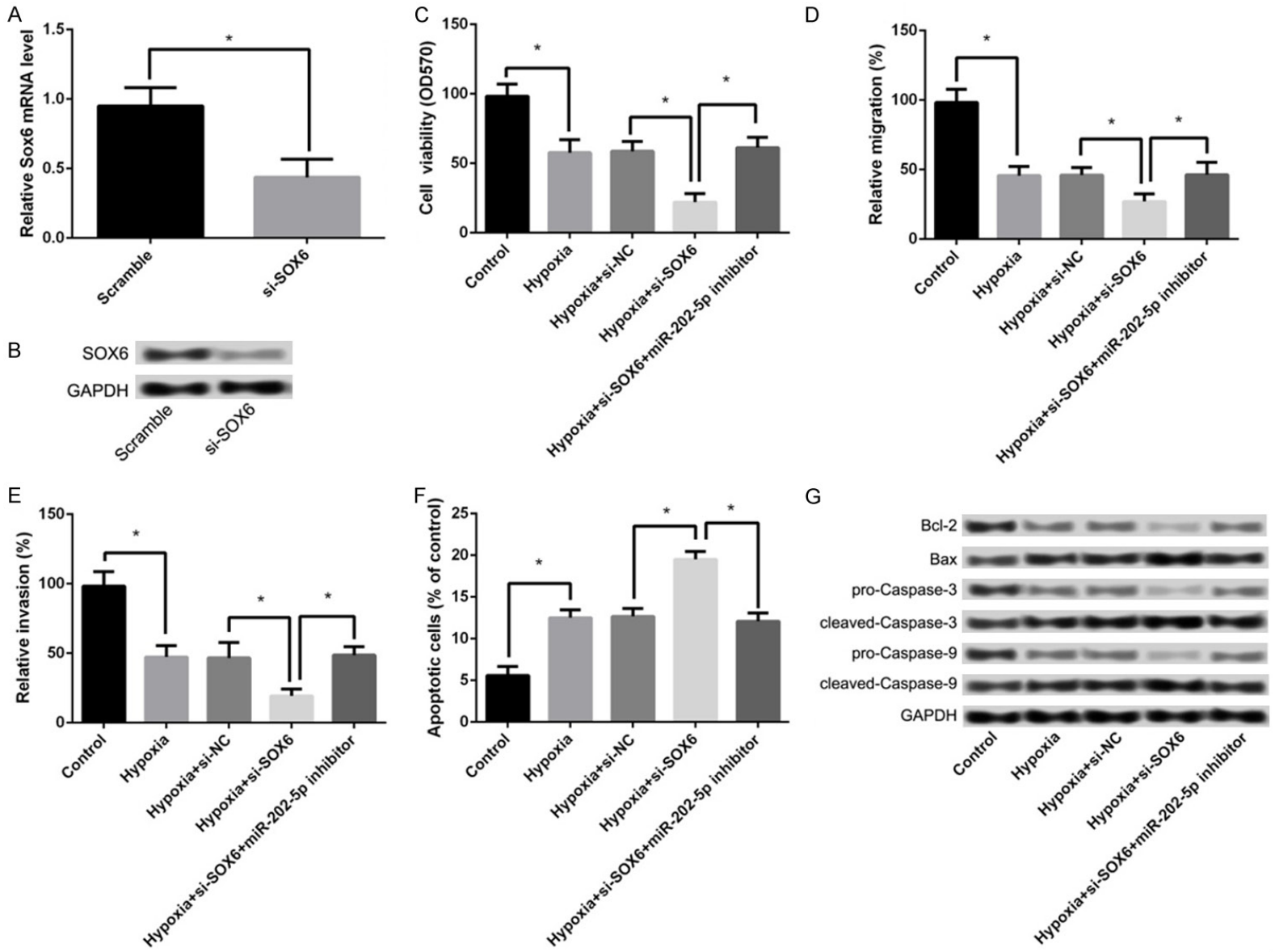

Figure 4. Knockdown of SOX6 aggravated hypoxia-induced myocardial damage, which was alleviated after inhibition of miR-202-5p. A and B: The mRNA and protein expression of SOX6 in different siRNAs transfection groups. C: Cell viability; D: Cell migration; E: Cell invasion; F: Cell apoptosis; G: The expression of apoptosis-related proteins. Data are expressed as mean ± SD. *, $P < 0.05$, **, $P < 0.01$.

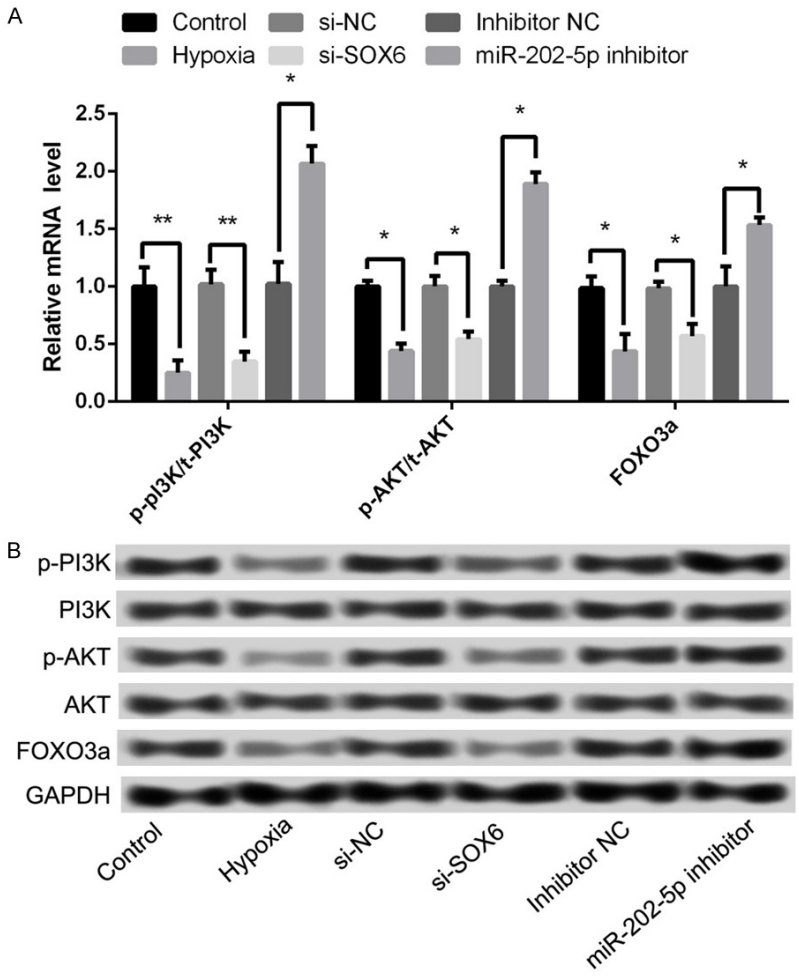

Figure 5. The mRNA and protein expression of p-PI3K/PI3K, p-AKT/AKT, and FOXO3a in myocardial H9c2 cells. Data are expressed as mean ± SD. *, P < 0.05, **, P < 0.01.

the suppression of miR-202-5p might alleviate the damage caused by hypoxia in H9c2 cells.

miR-202-5p targeted SOX6 and negatively regulated its expression

To explore the possible mechanism of miR-202-5p effects, we identified the target of miR-202-5p. Based on the information from TargetScan-Human, SOX6 was the potential target of miR-202-5p (**Figure 3A**). Moreover, the results of the luciferase reporter assay showed that the miR-202-5p mimic significantly inhibited the luciferase activity of SOX6-WT (P < 0.05; **Figure 3B**). Besides, the mRNA and protein expression levels of SOX6 in the miR-202-5p mimic group were significantly lower than those in the mimic NC group, while the levels were markedly higher in the miR-202-5p inhibitor group than those in the inhibitor NC group (P < 0.05; **Figure 3C**, **3D**). These results indicated that SOX-6 was the target of miR-202-5p and its expression was negatively regulated by miR-202-5p in H9c2 cells.

Knockdown of SOX6 aggravated hypoxia-induced myocardial damage, which was alleviated by inhibition of miR-202-5p

To investigate the relationship between miR-202-5p and SOX6 in H9c2 cells, we knocked down the expression of SOX6. The results showed that the mRNA and protein expression levels of SOX6 in si-SOX6 group were significantly lower than those in the scramble control group (P < 0.05; **Figure 4A**, **4B**). Compared to those in the hypoxia-treated cells, the cell viability (P < 0.05), migration (P < 0.05), and invasion ability (P < 0.05) of H9c2 cells further significantly decreased after knockdown of SOX6 (**Figure 4C-E**), while the cell apoptosis and the levels of BAX/BCL-2, active caspase-3, and active caspase-9 further increased (P < 0.05; **Figure 4F**, **4G**). Notably, the aggravating effects of SOX6 knockdown on the hypoxia-induced myocardial damage were reversed by concurrent inhibition of miR-202-5p (P < 0.05; **Figure 4C-G**).

Suppression of miR-202-5p activated the PI3K/AKT/FOXO3a pathway in H9c2 cells

The regulatory relationship between miR-202-5p and the PI3K/AKT/FOXO3a pathway in H9c2 cells was further investigated to elucidate the possible mechanism of action of miR-202-5p during myocardial ischemia (**Figure 5**). The results showed that the protein expression levels of p-PI3K, p-AKT, and mRNA and protein expressions of FOXO3a significantly decreased under hypoxia treatment (P < 0.05). The knock-

down of SOX6 also resulted in decreased expression levels of p-PI3K, p-AKT, and FOXO3a ($P < 0.05$). However, the opposite results were obtained after inhibition of miR-202-5p, showing that the mRNA and protein expression levels of p-PI3K, p-AKT, and FOXO3a significantly increased ($P < 0.05$).

Discussion

In this study, we investigated the role of miR-202-5p in regulating myocardial ischemia-caused injury. The results showed that miR-202-5p was significantly upregulated in hypoxia-induced H9c2 cells. The suppression of miR-202-5p significantly alleviated the hypoxia-induced damage in H9c2 cells through suppressing the cell apoptosis and promoting the cell viability, migration, and invasion ability. In addition, SOX6 was shown to be a direct target of miR-202-5p. The knockdown of SOX6 significantly aggravated the hypoxia-induced myocardial damage in H9c2 cells, which was alleviated by the inhibition of miR-202-5p. Besides, the knockdown of SOX6 inhibited the activation of the PI3K/AKT/FOXO3a pathway in H9c2 cells, whereas the inhibition of miR-202-5p activated the PI3K/AKT/FOXO3a pathway.

Revealing that SOX6 is a target of miR-202-5p is one of the important findings of our study. SOX6, belonging to the D subfamily of SOX transcription factors, is involved in biological progression, including cell proliferation, differentiation, and cell fate determination [14-16]. SOX6 has been found to play pathological roles in several diseases. Thus, it has been reported that SOX6 is implicated in sepsis-induced cardiac apoptosis through LPS-induced miR-499 inhibition [17]. Additionally, miR-202 promotes the progression of endometriosis through targeting SOX6 [18], and miR-208 promotes the proliferation of esophageal squamous cell carcinoma cells via suppression of SOX6 [19]. Although the relationship between miR-202-5p and SOX6 in H9c2 cells has not been previously investigated, considering the pathological roles of SOX6 in many diseases, we speculate that upregulation of miR-202-5p may promote the cell apoptosis and suppress the cell viability, migration, and invasion in hypoxia-induced myocardial H9c2 cells via targeting SOX6.

In addition, the regulatory relationship between miR-202-5p and the PI3K/AKT/FOXO3a pathway was further investigated to elucidate the regulatory mechanism of miR-202-5p in myocardial ischemia-caused injury. The PI3K/AKT pathway is a key pathway for mediating protective effects of SO_2 preconditioning against myocardial I/R injury in rats [20]. In a rat model of myocardial infarction, AKT/FOXO3a/SIRT1 have been found to mediate the cardioprotection by n-tyrosol against ischemic stress [21]. In addition, cyclic helix B peptide has been observed to repress the I/R-induced renal fibrosis through suppression of the PI3K/AKT/FOXO3a pathway [22]. Hydrogen sulfide can protect H9c2 cardiac cells from doxorubicin-induced cytotoxicity by regulating the PI3K/AKT/FOXO3a pathway [23]. Besides, the PI3K/AKT pathway has been confirmed as a key target of several natural compounds with a therapeutic potential, such as salidroside [24], berberine [25], and epigallocatechin gallate [26], which were able to attenuate the myocardial I/R injury. In our study, the inhibition of miR-202-5p resulted in increased expression levels of p-PI3K, p-AKT, and FOXO3a in myocardial H9c2 cells, while knockdown of SOX6 inhibited the expression of PI3K/AKT/FOXO3a pathway-related proteins. These data imply that the upregulation of miR-202-5p may target SOX6 to inhibit the activation of the PI3K/AKT/FOXO3a pathway, thus aggravating the myocardial ischemia-caused injury.

In conclusion, the data presented in this study revealed that miR-202-5p was upregulated in H9c2 cells during myocardial ischemia. The overexpressed miR-202-5p may aggravate the myocardial ischemia-caused injury, including the promotion of cell apoptosis and suppression of cell viability, migration, and invasion, in myocardial H9c2 cells by downregulating SOX6 to suppress the activation of the PI3K/AKT/FOXO3a pathway. Thus, miR-202-5p may be considered a potential target for the clinical treatment of myocardial ischemia.

Acknowledgements

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Disclosure of conflict of interest

None.

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